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Molecular biology of ethylene during tomato fruit development and maturation

Beatriz Cara a, James J. Giovannoni a,b,*

- ^a Boyce Thompson Institute for Plant Research, Ithaca, NY 14853, USA
- ^b United States Department of Agriculture-Agriculture Research Service (USDA-ARS), Robert W. Holley Center, Cornell University Campus, Tower Road, Ithaca, NY 14853, USA

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ABSTRACT

Important traits for complete ripening and consumer fruit quality preferences include development of aroma, flavor, color, texture, and nutritional quality. These attributes are influenced by the endogenously produced hormone ethylene in many fleshy fruits such as apple, avocado, banana, mango, pear and tomato. Even in species where endogenous ethylene seems to play little if any role as an endogenous regulator, exogenous ethylene will often promote ripening characteristics and can be the target of post-harvest strategies designed to accelerate, synchronize or delay ripening. In recent decades the YANG cycle for ethylene biosynthesis has been revealed and characterized at the molecular level with much of this important work done via the analysis of fruit systems. However, the genetic regulation that controls ethylene production at different developmental stages of fruits has only recently begun to be studied. Tomato has emerged as the primary model plant to further understand the molecular biology that controls ethylene synthesis and additional ripening regulators during fruit development. Here we summarize data pertaining to ethylene biology specifically as related to fruit maturation and including recent insights into genetic control of the ripening process prior to and controlling ethylene.

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1. Regulation of ethylene synthesis in tomato fruit

The phytohormone ethylene plays critical roles in many developmental events and environmental responses of plants. Climacteric fruits such as tomato, apple or pears, are characterized by a ripening-related increase in respiration and elevated ethylene synthesis to rapidly coordinate and synchronize ripening. In contrast, non-climacteric fruits such as strawberries, grapes or citrus, lack the respiratory peak associated with ripening. The reason for a respiratory climacteric is still poorly understood as nonclimacteric fruit manage ripening absent this change in physiology. Similar biochemical events often take place during ripening in both climacteric and non-climacteric fruits including color change. altered starch/sugar metabolism, fruit softening, textural modification, synthesis of aroma volatiles and increased susceptibility to pathogens. In addition, common genes regulating ripening in both types of fruits often show altered expression supporting the hypothesis that ethylene-dependent and ethylene-independent gene regulation pathways coordinate fruit maturation processes with primary regulators possibly conserved through evolution [1-5]. As reviewed elsewhere in this issue, the biochemical synthesis of ethylene was defined by the pioneering work leading to definition of the YANG cycle [6]. To summarize, the enzyme S-adenosylmethionine (SAM) synthase catalyzes adenosylation of the sulphur atom of methionine. SAM is then metabolized to 5'-methylthioadenosine (MTA), which is incorporated into the methionine cycle to recover the sulphur atom and 1-aminocyclopropane-1-carboxylic acid (ACC), the first compound of the pathway committed to ethylene biosynthesis. The enzyme catalyzing this reaction is ACC synthase (ACS) which is pyridoxal phosphate-dependent. Finally, in the presence of oxygen, ACC is converted to ethylene by ACC oxidase (ACO), originally defined as the ethylene-forming enzyme (EFE). Genes encoding ACS and ACO were originally identified via elegant studies employing maturing fleshy fruit [7,8].

Tomato has proven a highly useful model system for fruit development and ripening and is the system in which the role of ethylene during fruit ripening has been most thoroughly studied (for reviews see refs. [4–5]). Although several genes in the methionine biosynthesis pathway are responsive to ethylene during tomato fruit ripening [9], the ACS and ACO genes have been characterized most extensively. In tomato plants, nine genes encoding ACS (LeACS1A, LeACS1B, and LeACS2-8) have been described to date [10–18], and four are differentially expressed during fruit ripening: LeACS1A, LeACS2, LeACS4 and LeACS6 [19,11]. Barry et al. [19] proposed a model that explains the differential regulation of these genes during pre-climacteric (System 1) and climacteric (System 2) ethylene

^{*} Corresponding author at: Boyce Thompson Institute for Plant Research, Ithaca, NY 14853, USA. Tel.: +1 607 255 1414; fax: +1 607 254 1502.

E-mail address: jig33@cornell.edu (J.J. Giovannoni).

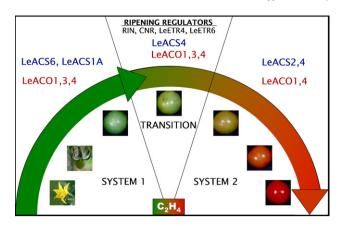


Fig. 1. Regulation of ethylene biosynthesis in tomato fruit development and ripening. During development (System 1) lower and auto-inhibitory ethylene is synthesized by LeACS1A,6 and LeACO1,3.4. At the transition stage, the ripening regulators indicated play critical roles. *LeACS4* is induced and a large increase of auto-catalytic ethylene starts, resulting in negative feedback on System 1. LeACS2,4 and LeACO1,4 are then responsible for the high ethylene production through System 2.

production of maturing tomato fruit (Fig. 1). Briefly, *LeACS*6 has been shown to be the main gene responsible for ACS and subsequent ethylene synthesis in green fruit (System 1), although expression of *LeACS1A* is also observed in these tissues. At the transition to ripening, expression of *LeACS1A* and *LeACS4* is induced and is further dependent on the RIN MADS-box transcription factor [20]. As a result of increased climacteric ethylene synthesis due to *LeACS1A* and *LeACS4* activation, *LeACS2* expression is also induced resulting in the auto-catalytic ethylene evolution characteristic of System 2 ethylene. High ethylene production occurs in the ripening fruit, resulting in negative feedback on the System 1 pathway and reduced *LeACS1A* and *LeACS6* expression.

Five genes encoding the ACO enzyme have been defined in tomato (*LeACO*1-5) and three of them (*LeACO*1, *LeACO*3, *LeACO*4) have been shown to be differentially expressed in fruit [10,11,13,14,19,21,22]. *LeACO*1 and *LeACO*4 accumulate in immature green stages and their expression levels were shown to increase dramatically at the onset of the climacteric burst and ripening. The expression of *LeACO*3 is induced but transitory at the breaker stage while *LeACO*1 and *LeACO*4 expression is sustained during ripening. The ripening-related induction of *LeACO*1 and *LeACO*4 expression is ethylene-dependent as defined in experiments where fruit where treated with the ethylene perception inhibitor 1-methylcyclopropene (1-MCP).

ACS and ACO genes have been characterized in many other fruits including but not limited to melon [23,24], apple [25–27], banana [28,29], pear [30], kiwifruit [31], peach [32,33] and persimmon [34]. In all cases examined at depth, these two families of enzymes have been shown to belong to multigene families and with members displaying unique expression patterns depending on developmental and environmental factors.

2. Transcriptional control of ethylene responsive genes in ripening fruit

From the mid 1980s to early 1990s numerous investigators demonstrated that ethylene regulates ripening in climacteric fruits by stimulating changes in gene expression. The hypothesis that ethylene mediated its ripening effects via regulated gene expression was both based on and shown through experiments where differential expression of mRNA transcripts and proteins was evaluated under conditions of exogenous ethylene addition or inhibition in wild-type fruits, natural ripening mutants and transgenic plants altered in expression of ethylene synthesis or response genes.

Examples of the best characterized ethylene regulated fruit genes reported in these studies include the previously described ethylene synthesis enzyme encoding genes ACS [35] and ACO [8]; the fruitspecific polygalacturonase (PG), which is involved in depolymerization of cell wall pectin during ripening [36,37], yet has little effect on fruit softening [38]; pectin methylesterase (PME), which provides accessibility to pectin by PG [39-41]; phytoene synthase (PSY), which catalyzes the rate limiting and highly regulated reaction from geranylgeranyl diphosphate to phytoene in the carotenoid biosynthesis pathway responsible for the pigmentation of many fruits and flowers including those of tomato [42]; or the ripening induced genes E4 and E8, whose functions are still uncertain, though the predicted peptides encoded by these genes show similarity to methionine sulphoxide reductase proteins and a dioxygenase with similarity to ACC oxidase, respectively [43,44]. These and many other ethylene responsive genes were recovered in multiple screens of ripening, ethylene treated and mutant fruits (reviewed in ref.

To better understand the mechanisms that control the expression of ethylene responsive genes during tomato ripening, the promoter regions of several of these genes were isolated and analyzed with the aim to identify functional regulatory motifs. The structure of the *LeACO1* gene promoter is well characterized [21]. The -1855 to -396 region of the promoter confers ethylene-dependent expression. It contains two repeat regions (RPT) with homology to ethylene responsive promoters of the ripening-specific genes 2A11 and E4. Several ethylene responsive regions (ERE; the 8 bp motif A(A/T)TTCAAA) and stress-related motifs (TCA; the 10 bp motif TCATCTTCTT) are also present in this promoter region. In contrast, the -396 region confers ethylene-independent expression.

Specific regulatory elements controlling the expression of ACS2 and ACS4 genes during tomato development and ripening were reported by Lincoln et al. It was shown that both promoters share a wound response element and the LeACS4 promoter contains a sequence with similarities to an anaerobiosis-responsive element (ARE) found in the alcohol dehydrogenase genes of maize. An analysis of the LeACS6 promoter has been recently reported [46]. The aim of this study was to identify the *cis*-elements responsible for the negative feedback control of ethylene at the transition from System 1 to System 2 during fruit ripening. The results localized putative cis-elements required for negative ethylene-response between -347 and -266 upstream from the translation start. Several LeACS6::GUS stable lines containing internal deletion of this region showed loss of response of the promoter to exogenous ethylene and provide a molecular explanation for the System 1 repression phenotype of this gene. Further analyses of the ciselements and the proteins that interact with them are needed to better understand the transcriptional regulation by ethylene of this gene. Furthermore, there is considerable evidence that regulation of ACS activity, through protein phosphorylation and turnover, also plays a critical role in the function of this enzyme suggesting that ethylene synthesis is regulated at several steps in the path from transcription to activity (for review, see ref. [47]).

The contrasting expression profiles of the *E*4 and *E*8 genes in response to ethylene make them attractive for analysis. Ethylene stimulates the transcription of the *E*4 gene in tomato fruit in response to both System 1 and System 2 ethylene. Indeed, every tissue analyzed for *E*4 expression results in expression upon exposure to ethylene and conversely, *E*4 can be found in virtually all tissues producing exogenous ethylene suggesting this gene is responsive to ethylene irrespective of tissue and developmental stage. In contrast, *E*8 is only induced in mature fruit (System 2-specific), indicating that ethylene regulation of this gene is both tissue-specific and developmentally regulated [48,49]. Analysis of the *E*4 promoter has shown that ethylene responsiveness of this

gene requires a minimum of two co-operative cis-elements, an upstream regulatory element between -150 and -121 bp and a downstream regulatory element between -40 and +65 [50]. An ERF (Ethylene Responsive Factor, also known as Ethylene Responsive Element-Binding Protein or EREBP) interacts with E4 and also the E8 promoter in a region that is necessary and sufficient for ethylene response in fruit from -1528 to -1100 as defined by promoter deletion studies of the E8 gene. This ERF is present in unripe fruit and its DNA-binding activity is reduced when treated with ethylene. This suggests that this ERF plays a repressor role in transcription. Another DNA-binding protein, E4/E8BP, has been identified that interacts with the downstream element of the E4 promoter and with a region regulating fruit-specific expression in the E8 promoter (from -1088 to -863 bp). A cDNA encoding a similar DNA-binding specificity was cloned and the encoded protein was named E4/E8BP-1. E4/E8BP-1 expression rates were found to be higher in fruit and increased during ripening, suggesting that E4/E8BP-1 plays a positive role on expression during ripening. The E8 gene promoter also contains a sequence from -409 to -263 required for expression during ripening but no binding element has been reported to date. In addition, enhancer elements that are active in leaves, anthers and pollen and other uncharacterized positive regulatory elements are present in the E8 promoter [44]. Taken together, these results imply that E4 and E8 may respond to a common mechanism for ethylene responsive gene expression but additional control systems limit E8 expression to the System 2 ripening fruit.

Polygalacturonase is only expressed in maturing tomato fruit tissue and its transcription is specifically activated during ripening. This characteristic expression pattern of the gene made the *PG* promoter very attractive for characterization with respect to fruit and ripening-specific regulation [51,52]. The different regions observed in the promoter and the 3′-flanking region showed complex interactions between positive and negative regulatory elements that tightly control gene expression. Whereas the -4822 to -1412 promoter region in conjunction with the 1.8 kb 3′ flanking region controls ripening-specific expression, the -1412 to -150 promoter region contains elements that direct spatial expression of the gene in the inner and/or outer pericarp.

The role of ethylene in *PG* regulation has been controversial. Regulation through an ethylene-independent mechanism was proposed based on experiments performed in transgenic ACC synthase antisense fruit [53]. However, Strit and Bennett [54] showed that these fruits produced low-level ethylene and as such *PG* belongs to a group of genes highly sensitive to basal levels of ethylene. The *PG* promoter also harbors ethylene-inducible elements with similarity to sequences found in the promoters of *F4* and *F8*.

With recent development of genomics tools for tomato and other species [55,56] and associated bioinformatic approaches for large scale data analysis and integration [57], it is becoming easier to analyze the expression profile of thousands of genes and metabolites in the same sample, opening the door to discovery of new regulatory networks not accessible with prior technologies. Alba et al. [55] identified 869 genes that are differentially expressed in tomato pericarp during ripening and 37% of them were shown to be under ethylene regulation as defined by differential expression in the *Never-ripe* ethylene receptor mutant. 72 of these genes were annotated as being related to signal transduction or transcriptional control. Moreover, expression analysis during fruit development revealed that 9 defined profiles of gene expression related to ripening and ethylene evolution. Such analyses provide considerable opportunities for further elucidating molecular regulatory networks impacting ethylene synthesis and response during ripening.

3. Ethylene signal perception and response during ripening

Phenotypic changes in response to ethylene are determined by three general steps: (1) the perception of the hormone, (2) the transduction of the signal through gene expression regulators and (3) the expression of genes and synthesis of proteins sensitive to the received ethylene signal. Knowledge of the components involved in ethylene perception and signaling has primarily been established in studies with *Arabidopsis thaliana* mutants altered in the seedling triple response (reviewed in refs. [58–65]). Dominant gain-of-function mutations in ethylene receptors resulted in reduced sensitivity to ethylene, while loss-of-function mutants in two or more of these redundant genes resulted in constitutive ethylene response. These results revealed the negative regulatory nature of these receptors in the ethylene signaling pathway [66.67].

The ethylene receptors are disulfide-linked dimers, endoplasmic reticulum (ER)-associated integral membrane proteins with similarity to bacterial two-component regulators, that include up to three domains termed the sensor, the kinase and the receiver (or response regulator) [68]. The sensor domain is localized in the Nterminal region of the protein and contains transmembrane stretches. It is responsible for perception of the ethylene molecule, dimerization and binding the necessary copper cofactor [69,70]. The kinase domain catalyzes autophosphorylation of a histidine residue from ATP. However, the sub-domains that define the catalytic core of the histidine kinase (HK) are not conserved in all the plant ethylene receptors. The histidine-phosphate group is transferred to an aspartate residue on the receiver domain that becomes active in signaling. This receiver element can be localized in the same sensor/HK protein or can be a separate protein. Both types of receiver elements have been observed in the described plant ethylene receptors (reviewed in ref. [71]).

In *Arabidopsis*, five receptors are responsible for perception of ethylene (ETR1, ETR2, ERS1, ERS2 and EIN4). They have been classified in two sub-families depending on predicted peptide structure. Subfamily 1 members (ETR1 and ERS1) contain three N-terminal membrane-spanning domains and the conserved histidine kinase domain, while subfamily 2 members (ETR2, ERS2 and EIN4) possess an additional transmembrane domain and the kinase domain lack one or more of the catalytic sub-domains, including the histidine that is autophosphorylated (ETR2, ERS2). Thus, the role of the histidine kinase activity remains under debate because of the lack of residues thought to be essential for its activity in subfamily 2 and due to a study [67] where an ers1:etr1 double mutant was rescued with a histidine kinase-inactivated form of ETR1.

In tomato there are three members of each sub-family [72–77]. LeETR1, LeETR2, and NR (Never-Ripe, also called LeETR3) are classified into sub-family 1. LeETR4, LeETR5, and LeETR6 are subfamily 2 members. A number of unique characteristics differentiate structure, expression patterns and regulatory mechanisms as compared to *Arabidopsis*. For example, while *Arabidopsis* has two receptors lacking the receiver domain (ERS1 and ERS2), only NR has this structure in tomato. Additionally, ethylene receptors in *Arabidopsis* have shown greater functional redundancy, while reduced expression of *LeETR4* resulted in constitutive ethylene response phenotypes in fruit and floral tissues. Interestingly, *NR* repression results in no discernable fruit phenotypes due to a compensatory increase in *LeETR4* expression [78].

The expression of the tomato ethylene receptors has been detected in all tissues analyzed, but they present distinct expression patterns throughout development and in response to differing environmental stimuli. *LeETR*1 and *LeETR*2 are expressed at constant levels in all tissues throughout development, while *NR*,

*LeETR*4, *LeETR*5 and *LeETR*6 are highly expressed in reproductive tissues (flowers and fruit) with a significant increase of *NR*, *LeETR*4 and *LeETR*5 in ripening fruits [71, and references therein].

Downstream of the Arabidopsis ethylene receptors acts the Constitutive Triple Response 1 (CTR1) gene which encodes a putative MAP-kinase kinase (MAPKKK or MAP3K) and through which all measured ethylene response flow. CTR1 is a negative regulator of ethylene response that likely interacts directly with receptor molecules to form a signaling complex [79-81]. While a single copy of CTR has been found in Arabidopsis, a small gene family was characterized in tomato (LeCTR1, LeCTR2, LeCTR3 and LeCTR4). LeCTR2 shares more similarity with Arabidopsis EDR1 (Enhanced Disease Resistance 1) than with CTR1 suggesting that LeCTR2 may not play a role in general ethylene response as appears to be the case with EDR1. LeCTR1, LeCTR3 and LeCTR4 are capable of complementing Arabidopsis ctr1 mutations suggesting that tomato may contain as many as three functional CTR1 homologs [2,3,9,82-84]. As with the ethylene receptors, all tissues evaluated express CTR1 genes and their mRNAs are differentially accumulated depending on tissue. LeCTR1 induction is associated with tissues at stages of development associated with increased ethylene, including fruit ripening, while LeCTR3 and LeCTR4 transcripts are not [2,3,82].

A MAP-kinase cascade has been implicated in the mediation of the ethylene response downstream of CTR1 in Arabidopsis [85], however direct interaction between CTR1 and a MAPKK remains to be demonstrated. Downstream of CTR1 acts the Ethylene Insensitive 2 (EIN2) [86] gene which encodes a protein with similarity to the Nramp family of metal ion carriers and for which loss-of-function mutations display ethylene insensitivity [87]. EIN2 is thought to play an important role in the signal transduction of other hormones such as abscisic acid [88,89], auxin [90], cytokinin [91] and jasmonate [92] and thus may represent a point of crosstalk between multiple hormone signaling pathways. The expression of LeEIN2 has been shown to be largely unchanging at different stages of fruit development, and is not regulated by ethylene [93]. The results from DNA gel-blot analysis pointed out that LeEIN2 is likely a single-copy gene as in Arabidopsis. Silenced fruits using a virus-induced gene silencing system had a delay in fruit development and ripening with reduced expression of ethylene-related and ripening-related genes, suggesting that LeEIN2 positively mediated ethylene signals during tomato development. Auxin-regulated genes were also reduced in silenced fruit which indicated that, as in Arabidopsis, LeEIN2 might be important for crosstalk between ethylene and auxin.

Downstream of EIN2, localized in the nucleus, is a family of *trans*-acting proteins termed EIN3 and EIL (or EIN3-like) transcriptional regulators [94,95]. EIN3, EIL1 and EIL2 have been shown to recognize specific motifs known as ethylene responsive elements (ERE) that are present within the promoters of several senescence and ripening-related genes and in the promoter region of the transcription factor Ethylene Response Factor 1 (ERF1) [94,95]. ERFs can interact with the ethylene responsive genes via binding to the 'GCC' box promoter elements [96,97]. ERF-type transcription factors are specific to plants and belong to the APETALA2 (AP2)/ERF family. Proteins encoded by this family have a highly conserved DNA-binding domain known as the AP2 domain [98].

Homologous of the *Arabidopsis EIL* gene termed *LeEIL*1–4 have been described in tomato [99–101]. *LeEIL*1–3 show the capability to complement the *Arabidopsis ein*3-1 mutant but their expression levels were not increased during fruit ripening or by exogenous ethylene treatment of leaves. In contrast, *LeEIL*4 exhibits ripening-induced expression (though ethylene inducibility of this gene remains to be shown). *LeEIL*1–3 antisense suppression studies revealed ethylene insensitivity in a dose-dependent manner. In

addition, over-expression of a *LeEIL1 GFP* fusion in the non-ripening *Nr* tomato mutant was able to partially restore ripening in concert with the expression of a subset of ethylene inducible genes [102].

Five tomato ERF genes (LeERF1-4 and LeERF3b) have been described to date [101,103,104]. Tomato LeERFs demonstrate binding capability to GCC-box elements. LeERF transcript accumulation studies indicated a specific pattern of expression for each gene, with LeERF2 (SI-ERF2) and LeERF3b displaying ripeningassociated transcript accumulation. While LeERF2 was induced during fruit ripening, LeERF3b accumulated before and declined sharply after the onset of ripening. Absence of *LeERF2* transcripts was observed in the tomato ripening mutants Nr, rin and nor. In contrast, LeERF3b mRNA is increased in low-ethylene tomato fruit containing an ACC oxidase sense-suppression transgene and in Nr mutant fruit. This result is consistent with a repressor function of LeERF3b with respect to the ethylene response. The LeERF2 promoter harbors a number of putative cis-regulatory elements including five putative EREs. It has been reported that LeERF2suppressed lines showed no visible phenotype while overexpressing lines showed enhanced ethylene response by exaggerated hook formation [103]. However, the role of ethylene in *LeERF2* expression levels during tomato fruit development has not been clarified and an effect on fruit ripening was not reported. Nevertheless, LeERF2 and putative homologues from additional plant species define a new ERF class termed class IV. Class IV ERFs contain a novel and highly conserved N-terminal motif of unknown function (MCGGAII/L), which deletion studies suggest is not required for either nuclear localization or GCC-box-binding activity [101]. Ethylene signal transduction components defined in Arabidopsis and tomato are summarized in Fig. 2.

Recent studies have contributed to our understanding of the complex regulation of ethylene perception and signal transduction [105,106]. The characterization in tomato of *Green-Ripe* (*GR*) and its

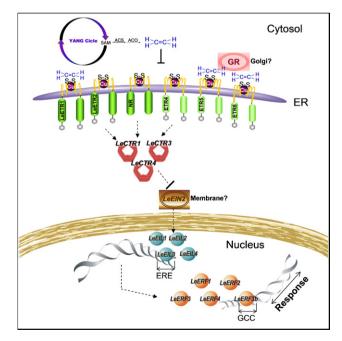


Fig. 2. Ethylene perception and signaling transduction pathway in tomato. Once ethylene is perceived by receptors (LeETR1,2,4-6, Nr) the signaling pathway is blocked with the participation of GR (second receptor in each pair is not named to indicate that homo- and hetero-dimers are likely to form). Downstream, CTR1-like proteins act also as negative regulators while LeEIN2 positively controls ethylene signaling and response. In the nucleus, LeEIL1-4 recognize EREs in the promoter of senescence and ripening genes including *LeERFs* which in turns can bind to GCC box in the promoter of ethylene responsive genes.

Arabidopsis homolog Reversion To Ethylene Sensitivity (RTE1) revealed a novel protein that negatively regulates ethylene response. In *Arabidopsis* it was shown that ethylene insensitivity in the *etr*1-2 gain-of-function mutant could be restored by mutation in RTE1. The tomato GR mutant fails to fully ripen as a consequence of selectively reduced ethylene responsiveness in fruit and floral tissues [107]. This inhibition is due to an ectopic-expression gain-of-function mutation resulting from a 334-bp deletion in the 5'-flanking region of the GR protein. Further analysis of the Arabidopsis ethylene receptors and RTE1 interactions has revealed the dependency of this protein on the ETR1 N-terminus to negatively regulate ethylene response, though the histidine kinase and receiver domains are not required [108]. In agreement with previous evaluations of RTE1 and GR as membrane proteins with putative binding sites for divalent cations (specifically copper), GFP-RTE1 fusion studies confirmed localization to the Golgi and with lesser signal in the ER. Phylogenetic analysis illustrated that GR and RTE1 are conserved in plants, animals and protists. Because ethylene receptors require copper for activity, it is logical to hypothesize that GR and RTE1 functions might be related to receptor-copper interactions. Furthermore, the tissue-dependent ethylene response observed in the Gr mutant implies that components of the ethylene signaling pathway may be unique in the fruit of tomato [105].

4. Initiation of fruit ripening and ethylene production

Recent discoveries have broadened our understanding of the regulatory mechanism that precede and regulate ethylene induction during ripening. For example, a role for the LeETR4 and LeETR6 ethylene receptors in modulating the timing of ripening has been demonstrated [109]. LeETR4 and LeETR6 proteins are degraded in response to ethylene application resulting in accelerated fruit ripening. The authors proposed a model in which ethylene receptor content is a major determinant of ripening initiation. Specifically, as the receptors are negative regulators of ethylene signaling, receptor depletion would result in a progressive increase in hormone sensitivity triggering ripening when a specific threshold of receptor levels is reached. In this regard, the receptors themselves serve a role in the development of ripening competency during fruit maturation.

Additional elements independent of ethylene are known to play key roles in ripening initiation. The *ripening-inhibitor* (*rin*), *Colorless non-ripening* (*Cnr*) and *non-ripening* (*nor*) tomato mutants provide valuable information in this regard [4,5]. The fruit produced by these mutants fail to display normal ripening phenotypes including ethylene synthesis, increased respiration, carotenoid accumulation, softening, and aroma volatile production [110,111]. Moreover, *rin*, *Cnr* and *nor* are not able to ripen in response to exogenous ethylene application, though ethylene-regulated gene expression is partially restored indicating retention of ethylene sensitivity [19,20,110,112,113,114,100]. Thus, these genes have ethylene-independent functions playing important roles in the regulation of fruit ripening and ethylene synthesis and which operate prior to activation of the ethylene synthesis and signaling pathways.

The *rin* locus encodes a MADS-box transcription factor termed LeMADS-RIN and is a member of the SEPALATA subfamily of MADS genes [20], while the *Cnr* mutation resulted from a dominant epigenetic alteration in the promoter of a SQUAMOSA promoterbinding protein (SBP) [112]. It is known that MADS-box genes act together in multimeric complexes [115] and SBP proteins have been shown to directly regulate the expression of MADS-box genes. It is plausible to anticipate that other tomato MADS-box genes may interact with *RIN* in the control of fruit ripening and *CNR* may act to directly influence *RIN* expression or the expression of other MADS-box genes which may be participating in fruit ripening.

In agreement with the theory of ethylene-independent ripening functions mentioned above, a fruit-specific homolog of *LeMADS-RIN* was identified in strawberry [20], a non-climacteric fruit. This finding suggests the possibility that ripening transcription factors may represent a conserved function in the regulation of ripening in both climacteric and non-climacteric species [4,20]. A number of candidates gene that may play such a role have been identified by cross-species expression analysis based on prevalence of homologous transcription factor transcripts in EST collections [116].

The tomato *TDR*4 gene was isolated based on homology to *Arabidopsis* MADS-box genes and encodes a SQUAMOSA MADS-box transcription factor whose function in tomato has been associated with cell wall structure and metabolism [117–119]. It has been proposed that *TDR*4 is an orthologue of *Fruitfull* (*FUL*) of *Arabidopsis*, which is a negative regulator of the fruit dehiscence-related *Shatter Proof* (*SHP*) genes [120]. Expression of *TDR*4 is low during fruit development and induced at the onset of ripening. Consistent with a role in ripening, expression of *TDR*4 is down-regulated in the *Cnr*, *rin* and *nor* mutants [119]. Thus, it has been proposed that CNR, RIN and TDR4 are all part of the same regulatory network [119,121].

It will be especially interesting to identify the direct gene targets of CNR, RIN and TDR4 and determine the metabolic pathways that they regulate during ripening both through ethylene and out of the realm of ethylene control. For example, are the System 2 ethylene synthesis genes direct targets of these regulators and what is the regulatory relationship among these genes? Seymour et al. [121] suggest a regulatory cascade in which *CNR* requires RIN and is necessary for *TDR4* transcription. Is this a simple linear regulatory pathway or is it modulated by additional regulators? What additional regulatory genes exert their control over ripening and will the systems defined in tomato prove to be similar in the fruits of additional species?

5. Futures perspectives

Recent developments in genomics and the application of these tools to fruit species (especially tomato) is likely to provide a rapidly expanded view of ethylene response and ripening control in coming years. Microarray studies have begun to define the ethylene and ripening transcriptomes [56] and further comparison with expression in mutants such as rin and nor should allow for the distinction of the ethylene and non-ethylene regulated ripening transcriptomes. While most tomato ripening mutants described to date represent spontaneous mutation events, induced mutant populations are being developed and organized (http://zamir.sgn.cornell.edu/mutants/) and should provide new opportunities to understand ripening control. The recent finding of GR as a gene with the capability to alter ethylene response specifically in the fruit provides exciting possibilities regarding elucidation of mechanisms of fruit-specific ethylene response, an area that is currently poorly understood.

Following on in relation to opportunities through mutagenesis, exciting opportunities exit through the potential use of TILLING (Targeting Induced Local Lesions IN Genomes) which has been developed and successfully applied in *Arabidopsis* [122] and other plants [123]. The aim of this non-transgenic approach focuses on the discovery of new mutant alleles typically at a single nucleotide and based on existing DNA sequence for genes of interest (for details see ref. [124]). In tomato there exist EMS (ethylmethane sulfonate)-mutagenizated populations both in Micro-Tom [125] and the M82 background [126]. Furthermore, several tomato TILLING platforms are in progress including the Franco-Italian effort coordinated by the EU-SOL project (http://www.eu-sol.net) and others are in development around the world. One of the distinct advantages of TILLING includes the capability to detect

mutations in essential genes that may prove lethal when approached by less subtle methodologies such as antisense or RNAi.

The post-transcriptional control of expressed genes by small RNAs (sRNA) such as microRNAs (miRNA) or small interfering RNAs (siRNA) during tomato fruit development and ripening is an emerging field. The existence of fruit-specific sRNAs has been reported [127,128] but a role of this regulation system in ethylene synthesis, transduction or response remains to be elucidated. The creation of a database (http://ted.bti.cornell.edu/digital/sRNA/) for public access to sRNAs from tomato fruit and leaf tissues represents and excellent introduction to the study of sRNAs in maturing fruit.

The enormous effort made to create tomato introgression lines (ILs) populations of wild-species such as *Solanum pennellii* [129] and *Solanum habrochaites* [130] in a cultivated *Solanum lycopersicum* background is driving the efficient mapping of hundreds of QTLs related to important fruit quality and yield traits and will facilitate cloning of some of these genes [131–135]. Due to the important role that ethylene plays in many of these traits, some of these QTLs may well prove to represent ethylene synthesis, response or related regulatory loci. Indeed, cloning of such loci could lead to the discovery of new components of ethylene signaling and response pathways and contributors to regulatory networks with other metabolic pathways affecting fruit ethylene response, ripening and quality.

The tomato genome is currently being sequenced by an international consortium (www.sgn.cornell.edu) and further advances in understanding the molecular biology of ethylene regulation will certainly be impacted by this effort and transcriptional profiling which will provide expression annotation to the genome sequence. For example, there will be opportunities to look at promoter sequences of ethylene responsive and ripening genes to search for common regulatory elements associated with ethylene response and/or ripening control. Finally, the development of large scale sequence and expression data for additional fruit species will promote opportunities for translational biology regarding fruit development and ethylene response to be applied to additional crop species.

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